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Citation for published version:

Forns, X, Maluenda, MD, López-Labrador, FX, Ampurdanès, S, Olmedo, E, Costa, J, Simmonds, P, Sánchez-Tapias, JM, Jimenez De Anta, MT & Rodés, J 1996, 'Comparative study of three methods for genotyping hepatitis C virus strains in samples from Spanish patients', *Journal of Clinical Microbiology*, vol. 34, no. 10, pp. 2516-21.

Link:

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Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Clinical Microbiology

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Comparative Study of Three Methods for Genotyping Hepatitis C Virus Strains in Samples from Spanish Patients

XAVIER FORNS,¹ MARÍA D. MALUENDA,¹ FRANCESC X. LÓPEZ-LABRADOR,¹ SERGI AMPURDANÈS,¹
EVA OLMEDO,¹ JOSEP COSTA,² PETER SIMMONDS,³ JOSÉ M. SÁNCHEZ-TAPIAS,^{1*}
MARÍA T. JIMÉNEZ DE ANTA,² AND JUAN RODÉS¹

Liver Unit¹ and Laboratory of Microbiology,² Hospital Clínic i Provincial, University of Barcelona, 08036 Barcelona, Catalonia, Spain, and Department of Medical Microbiology, University of Edinburgh Medical School, EH8 9AG Edinburgh, United Kingdom³

Received 27 December 1995/Returned for modification 29 March 1996/Accepted 22 July 1996

Hepatitis C virus (HCV) genotypes may be investigated by a variety of laboratory methods that target different parts of the HCV genome and have various degrees of technical difficulty. Since the choice of a particular method is difficult, we compared the performance of (i) a type-specific PCR with type-specific primers from the core region, (ii) molecular hybridization of the PCR-amplified 5' noncoding region to type-specific probes, and (iii) identification of type-specific antibodies against epitopes of nonstructural region 4 by enzyme-linked immunosorbent assay (ELISA). One hundred fifty-one patients with biopsy-proved chronic hepatitis and HCV RNA in serum were investigated. The HCV genotype was identified in 99%, 100%, and 85% of the cases by type-specific PCR, probe hybridization, and ELISA, respectively. The type-specific PCR disclosed infection with type 1a in 3%, type 1b in 74%, and type 3a in 4% of the cases and suggested infection with two or more HCV types, including 2a/2c and 2b, in the remaining 18%. Apparently mixed infections were more prevalent in patients with past intravenous drug use ($P < 0.001$), but cloning and sequencing of PCR products did not confirm a mixed infection in any of the four cases investigated. Concordant results were obtained by the three procedures with virtually all of the samples in which the type-specific PCR revealed a single HCV genotype. Type-specific hybridization and ELISA usually recognized the genotype producing the strongest DNA band in samples in which type-specific PCR suggested a mixed infection. In conclusion, the three procedures evaluated in this study are reliable for investigation of HCV genotypes. Type-specific PCR provides information about HCV subtypes, but a mixed infection detected with this method should be interpreted with caution.

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus with marked genetic heterogeneity, as shown by sequence analysis of isolates from different parts of the world (2, 3). As in infections with other RNA viruses, the genetic heterogeneity of HCV is probably related to replication errors introduced by the RNA-dependent RNA polymerase (7, 16, 23, 36).

On the basis of comparison of nucleotide and predicted amino acid sequences of different regions of the HCV genome, at least six major genotypes have been described (2–4, 24, 29, 31, 35) and a new system for nomenclature was recently agreed by several research groups (28). In this new nomenclature, the classification of HCV into different types and subtypes is based on sequence homology of at least two regions of the genome and confirmed by phylogenetic tree analysis.

Recent data suggest that the natural course of the disease (22) and the response to interferon therapy (9, 37, 39) may be influenced by the genotype of the virus infecting each individual patient. Therefore, HCV typing may become an important tool in clinical practice, mainly for design of therapeutic strategies.

Several methods have been developed to identify HCV genotypes, including PCR amplification of HCV cDNA with type-specific primers (24), restriction fragment length polymorphism analysis of PCR amplicons (8, 21, 30), molecular

hybridization of type-specific probes to PCR-amplified HCV cDNA (13, 35), genome amplification and sequencing (2, 4, 36), and identification of type-specific antibodies by enzyme-linked immunosorbent assay (ELISA) (20, 31). Since all of these techniques focus on different regions of the HCV genome, it may be important to explore whether genotyping by different methods produces concordant results or not. On the other hand, the economic cost, the degree of technical difficulty, and the amount of information provided may vary considerably from one procedure to another, making a choice difficult.

We report here the results obtained with a large series of Spanish patients with chronic hepatitis C in whom the HCV genotype was investigated by three procedures focusing on different regions of the HCV genome and involving distinct methods, such as a type-specific PCR, molecular hybridization, and ELISA for type-specific antibodies.

MATERIALS AND METHODS

Sera from 151 consecutive HCV-infected Spanish patients with biopsy-proved chronic hepatitis referred for antiviral therapy were studied. Etiological factors other than chronic HCV infection were ruled out in all cases. All patients had had elevated serum aminotransferases for at least 6 months, had a positive test for anti-HCV antibodies (third-generation ELISA [Ortho Diagnostics, Raritan, N.J.]), and had HCV RNA in serum by reverse transcription nested PCR with primers from the 5' noncoding region (5'NCR) of the HCV genome (12, 33). The mean age of the patients was 44 (range, 18 to 64) years. Ninety-one were male, and 60 were female. Fifty-one patients had a history of blood transfusion, 21 had a history of intravenous drug use (IVDU), and the possible source of infection was not determined in the remaining 79. In accordance with recently proposed criteria (11), the histological diagnoses were mild chronic hepatitis in 60 cases, moderate chronic hepatitis in 54, and severe chronic hepatitis in 37.

* Corresponding author. Mailing address: Liver Unit, Hospital Clínic i Provincial, Villarroel 170, 08036 Barcelona, Catalonia, Spain. Phone: 34 3 227 5400, ext. 2208. Fax: 34 3 227 5454. Electronic mail address: sanchez@medicina.ub.es.

TABLE 1. Sequences of primers used for HCV cDNA amplification by type-specific PCR

Designation	Sequence (type)
Universal primers for 1st PCR round	
256	5' CGCGCGACTAGGAAGACTTC 3'
256V	5' CGCGCGACGCGCAAACTTC 3'
186	5' ATGTACCCCATGAGGTCGGC 3'
Universal primers for 2nd PCR round	
104	5' AGGAAGACTTCCGAGCGGTC 3'
104V	5' CGTAAACTTCTGAACGGTC 3'
Type-specific primers for 2nd PCR round	
132	5' TGCCTTGGGGATAGGCTGAC 3' (1a)
133	5' GAGCCATCCTGCCACCCCA 3' (1b)
134m ^a	5' GCCCATGAAGGGCGAGAAC 3' (2a/2c)
135	5' ACCCTCGTTTCCGTACAGAG 3' (2b)
339	5' GCTGAGCCAGACCGGTCT 3' (3a)

^a This primer was designed by modification of primer 134 (24) as described by Silini et al. (27).

Pretherapy serum samples were obtained under appropriate conditions to improve preservation of RNA and immediately stored at -40°C until tested (10). The HCV genotype was examined by three methods: (i) a type-specific PCR, (ii) hybridization of type-specific probes to DNA obtained by PCR amplification of HCV cDNA, and (iii) identification of type-specific antibodies.

HCV RNA extraction and cDNA synthesis. For detection of HCV RNA in serum and for genotyping studies involving PCR, RNA was extracted from 180 μl of serum by the acid guanidinium isothiocyanate-phenol-chloroform method (6), precipitated with isopropanol, and rinsed with 70% cold ethanol. The RNA pellet was resuspended in 25 μl of diethylpyrocarbonate-treated water. cDNA was synthesized from 7 μl of RNA with 300 U of Moloney murine leukaemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, Md.) in the presence of random primers and 20 U of RNase inhibitor (Promega, Madison, Wis.). The guidelines of Kwok and Higuchi (18) were strictly observed to prevent carryover contamination, and appropriate negative controls for RNA extraction, cDNA synthesis, and PCRs were routinely included in each PCR round.

Type-specific PCR. Genotyping with type-specific primers from the core region of the HCV genome was performed by type-specific PCR as described by Okamoto et al. (24), with the following modifications: (i) use of universal and type-specific primers for type 3a (25), (ii) use of a modified primer for types 2a and 2c in accordance with nucleotide sequence differences observed between Mediterranean and Japanese HCV isolates (17, 27), and (iii) addition of 500 μM tetramethylammonium chloride (TMAC; Sigma-Aldrich, Madrid, Spain) to the PCR mixture to increase the specificity of annealing (15). Reactions were carried out in a Perkin-Elmer Cetus thermal cycler with *Taq* DNA polymerase (GIBCO BRL). The first round of amplification was performed with 7 μl of cDNA by using a combination of two sense primers (256 and 256V) and one antisense primer (186) (Table 1). The reaction consisted of 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 7 min. One microliter of the PCR products was reamplified by a second PCR round (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles plus 72°C for 7 min in the final cycle) with a mixture of two sense primers (104 and 104V) and five antisense primers (132, 133, 134m, 135, and 339) (Table 1). Type-specific PCR products were identified by electrophoresis on 3% NuSieve and 1% SeaKem agarose (FMC Bioproducts, Rockland, Maine) after ethidium bromide staining. A ϕ -X174 DNA *Hinf*I digest (Promega) was used as a molecular weight marker. Serum samples giving more than one genotype-specific DNA band were reanalyzed, and the presence of a mixed infection was accepted upon agreement by two independent observers of the gels. When a type-specific PCR suggested a mixed infection, the genotype represented by the most intense DNA band was considered the predominant genotype.

Type-specific hybridization. Genotyping by hybridization with type-specific probes was performed as described by Gerotto et al. (13), with minor modifications. Briefly, 7 μl of cDNA was amplified with primers from the well-conserved 5'NCR (12). The reaction consisted of 35 cycles of 95°C for 1 min, 46°C for 1 min, 72°C for 1 min and a final extension for 7 min. Amplified DNA (10 μl) was denatured with NaOH and applied to a prehumidified nylon membrane (Boehringer, Mannheim, Germany) with a dot-blotting manifold and then fixed by baking for 30 min at 120°C . Membranes were prehybridized for 60 min at 42°C . Prehybridization buffer was composed of $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% blocking reagent (Boehringer), 0.1% *n*-lauryl sarcosinate, and 0.02% sodium dodecyl sulfate (SDS). Hybridization was performed

by incubating the membranes overnight at 42°C in the same buffer, with type-specific 5' end digoxigenin-labelled probes synthesized from the 5'NCR (30 pmol/ml). The sequences of the probes used for type-specific hybridization were as follows: type 1, 5' CGCTCAATGCCTGGAGAT 3'; type 2, 5' CACTCTAT GCCCGGCCAT 3'; type 3, 5' CGCTCAATACCCAGAAAT 3'. Washing was performed as follows: 10 min with $6\times$ SSC-0.1% SDS at room temperature (twice), 10 min with $3\times$ SSC-0.1% SDS at room temperature (twice), and 15 min with $1\times$ SSC-0.1% SDS at 55°C (twice). Thereafter, filters were incubated with an antidigoxigenin-alkaline phosphatase conjugate and colorimetric detection was performed with nitroblue tetrazolium salt (75 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate salt (50 mg/ml; Boehringer).

Detection of type-specific antibodies by ELISA. Type-specific antibodies to HCV were identified by ELISA with branched oligopeptides deduced by epitope mapping of the nonstructural region 4 (NS4) protein as described previously (31). In this assay, plastic wells were coated with peptides (100 ng/ml) corresponding to each of the three HCV types, 1, 2, and 3. Test samples at a 1:40 dilution were incubated in individual wells in the absence and in the presence of a 100:1 molar excess of type heterologous peptides (1 plus 2, 1 plus 3, or 2 plus 3) in solution. The aim of this strategy was to ensure specificity by preventing binding to the wells of antibodies against epitopes shared by the HCV types present in the blocking solution. A sample was considered nonreactive when no reaction occurred at either a 1:40 or a 1:10 dilution. A sample was considered nontypeable when reactivity appeared with the unblocked peptides and no reactivity appeared with the peptides in solution (31).

All of the nonreactive and nontypeable samples and the 10 typeable samples were tested for the presence of antibodies targeting different regions of HCV; i.e., NS4 (c100/5-1-1 peptides), NS3 (c33c antigen), core (c22 peptide), and NS5 (NS5 antigen), with a third-generation recombinant immunoblotting assay (Chiron RIBA HCV 3.0; Ortho Diagnostics).

5'NCR cloning and sequencing. Cloning and sequencing analysis of the 5'NCR were carried out with six samples that were selected on the basis of data provided by a type-specific PCR. The HCV 5'NCR was amplified from cDNA as described above and cloned into a pBluescript SK(+) plasmid vector (Stratagene Cloning Systems, La Jolla, Calif.). The DNA sequence was determined with at least 10 clones from each patient by using the AutoRead DNA Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) and an A.L.F. automatic DNA sequencer (Pharmacia Biotech). The HCV genotype present in each clone was recognized by identification of previously described type- and subtype-specific sequence polymorphisms of the 5'NCR (8).

Statistical analysis. Differences between proportions were analyzed with the chi square test.

RESULTS

Genotyping by type-specific PCR. The distribution of HCV genotypes identified by the three methods compared in this study is shown in Table 2. Type-specific DNA bands obtained with the type-specific PCR were identified by their distinct sizes in 149 (99%) of the 151 cases. Infection by a single type was detected in 121 cases (80%), and extra bands, suggesting coinfection by two or more types, were present in 28 cases (19%), including 7 with infection by subtypes of the same genotype.

Among the samples in which an apparent mixed infection was identified by the type-specific PCR, we detected two types of HCV in 15 cases, three types in 12 cases, and four types in

TABLE 2. Results of different HCV typing methods for 151 patients with chronic hepatitis C

HCV type or subtype	No. of samples typed by:		
	Type-specific PCR	Type-specific hybridization	ELISA
1	111	124	103
1a	5		
1b	106		
2		5	7
2a/2c			
2b			
3	10	16	14
3a	10		
Mixed	28	6	4
Not identified	2		23

TABLE 3. Distribution of HCV types among 121 patients infected by a single HCV type as assessed by type-specific PCR and relationship with results obtained by type-specific hybridization and by ELISA

HCV type determined by type-specific PCR	Total no. of samples	HCV type(s) (no. of samples) determined by:	
		Type-specific hybridization	ELISA
1a	5	1 (5)	1 (5)
1b	106	1 (105)	1 (87)
1b		1 + 2 (1)	1 + 2 (2)
1b			NR ^a or NTS ^b (17)
3a	10	3 (9)	3 (8)
3a		3 + 1 (1)	1 (1)
3a			NTS (1)

^a NR, nonreactive sera.

^b NTS, non-type-specific antibodies detected.

1 case. Genotypes 2a/2c and 2b, which were not detected in samples with single infections, were found in 20 (71%) of 28 samples with apparently mixed infections.

Predominance of one of the coexisting types was observed in 22 of the 28 cases with apparently mixed infections; the predominant type was genotype 1a in 3 cases, genotype 1b in 4, genotypes 2a/2c in 6, genotype 2b in 1, and genotype 3a in 8. There were significant differences in the frequency of distribution of genotypes between patients with single and apparently mixed infections. Genotype 1b was detected in 88% of patients with single infections but it was the predominant type in only 14% of those with apparently mixed infections ($P < 0.001$). In contrast, genotypes 2 and 3 were rare or absent in samples in which a single genotype was identified but very common in those with apparently mixed genotypes. The epidemiological features of patients with single infections and those with apparently mixed infections were significantly different, since a history of IVUD was rare in the former (5.8%) but common in the latter (50%) ($P < 0.001$).

Genotyping by type-specific hybridization. All of the sera could be typed by type-specific hybridization. Infection with a single genotype was detected in 145 (96%) of 151 patients, and mixed infections were observed in only 6 patients (4%) (Table 2).

Genotyping by ELISA. The infecting genotype was identified by ELISA in 128 samples (85%). Single infections were found in 124 cases (82%), and mixed infections were found in 4 (2.6%). The HCV genotype was not identified by ELISA in 23 samples, of which 13 were reactive but nontypeable and 10 were nonreactive. Among these 23 samples, the type-specific PCR showed type 1b in 18 cases, type 3a in 1, and mixed infections in 4. Thus, the distributions of HCV genotypes (as identified by the type-specific PCR) in samples with typeable and nontypeable infections by ELISA were similar.

The 13 reactive but nontypeable, 5 of the 10 nonreactive, and the 10 typeable samples studied showed strong reactivity against all of the Chiron RIBA HCV 3.0 peptides, including those from NS4. The remaining five nonreactive samples were strongly reactive for c33c and c22p but did not react against NS4 or NS5 peptides.

Comparison of methods. HCV genotype identification was achieved in 99% of the cases by type-specific PCR, in 100% by type-specific hybridization, and in 85% by ELISA. The results provided in each individual case by the three methods were remarkably concordant when only the predominant genotype detected by type-specific PCR was considered.

Among the 121 samples in which a type-specific PCR disclosed infection by a single HCV genotype, type-specific hybridization gave concordant results in more than 95% of the cases (Table 3). Remarkably concordant results were also obtained by ELISA, although this technique gave no information on 18 of these 121 patients. A major disagreement between the results obtained by ELISA and the other two procedures occurred for only one patient, in whom the ELISA recognized HCV type 1 whereas the type-specific PCR and type-specific hybridization recognized type 3.

Type-specific hybridization identified a single genotype in 18 of the 21 samples in which the type-specific PCR suggested mixed infections with different major genotypes. In these cases, type-specific hybridization identified the genotype that appeared predominant in the type-specific PCR analysis. In the seven samples in which the type-specific PCR disclosed two subtypes of the same genotype (1a plus 1b or 2a/2c plus 2b), the common type (1 or 2) was identified by type-specific hybridization. A partial discrepancy between the results obtained by the type-specific PCR (2a/2c plus 2b) and type-specific hybridization (2 plus 1) occurred in only one case (Table 4).

Genotyping by ELISA and by type-specific PCR produced remarkably concordant results with samples in which the type-specific PCR disclosed apparently mixed infections. Among the 22 samples in which the type-specific PCR suggested the predominance of one genotype, ELISA identified the predominant genotype in 18 cases. In two samples with which the type-specific PCR produced more than one DNA band, ELISA identified two concordant genotypes. No major disagreement among the ELISA, the type-specific PCR, and type-specific hybridization was observed in any case (Table 4).

Cloning and sequencing of the 5'NCR. Two samples with a single infection (type 3a) and four samples with apparently mixed infections (1a plus 1b in two cases, 1b plus 3a in one, and

TABLE 4. HCV types in 28 patients with mixed infections identified by type-specific PCR and relationship with results of typing by type-specific hybridization and ELISA^a

HCV type(s) determined by type-specific PCR	Total no. of samples	HCV type(s) (no. of samples) identified by:	
		Type-specific hybridization	ELISA
1b + 1a	2	1 (2)	1 (1) NR ^b (1)
1b + 2a/2c	1	1 (1)	1 + 2 (1)
1b + 2b	1	1 (1)	1 (1)
2a/2c + 1b	1	2 (1)	2 (1)
2a/2c + 2b	1	2 + 1 (1)	2 (1)
3a + 1b	2	3 + 1 (1)	NTS ^c (2)
3a + 1b		3 (1)	
3a + 2b	1	3 (1)	3 (1)
1a + 1b + 2b	3	1 (3)	1 (3)
2a/2c + 1b + 2b	4	2 (2)	2 (3)
2a/2c + 1b + 2b		2 + 1 (2)	2 + 1 (1)
2b + 1a + 1b	1	2 (1)	2 (1)
3a + 1b + 1a	1	3 (1)	NR (1)
3a + 1b + 2b	3	3 (3)	3 (3)
3a + 1a + 1b + 2b	1	3 (1)	3 (1)
1a + 1b	3	1 (3)	1 (3)
1b + 2b	2	1 (2)	1 (1)
1b + 2b			NTS (1)
2a/2c + 2b	1	2 (1)	2 (1)

^a Bold characters identify the predominant genotype in type-specific PCR experiments.

^b NR, nonreactive sera.

^c NTS, non-type-specific antibodies detected.

1a plus 1b plus 3a in another) according to the type-specific PCR were studied. A single HCV type was identified in 10 to 13 clones sequenced from each patient. In the two cases in which the type-specific PCR suggested a 1a-plus-1b coinfection, 10 type 1a sequences were identified in one case and 10 type 1b sequences were identified in the other. Only type 3a sequences were identified in the clones analyzed from the two samples with apparently mixed infections with type 3a and other genotypes and from the two samples with a type 3a single infection.

The genotype identified by cloning and sequencing always agreed with the genotype that produced the most intense DNA band in type-specific PCR experiments.

DISCUSSION

Identification of the infecting virus genotype has become relevant in the investigation of many aspects of HCV infection, including epidemiology, pathogenesis, and response to antiviral therapy. Several laboratory procedures aimed at identifying the HCV genotype have been described. A PCR with type-specific primers from the core region, as described by Okamoto et al. (24, 25), has been extensively used. Other assays based on type-specific PCR amplification with type-specific primers from other regions of the HCV genome, such as NS5, have also been described (5). Inno-LiPA is also a widely used assay that is based on the hybridization of amplified cDNA from the 5'NCR to specific oligonucleotide probes immobilized on a membrane strip (34). HCV can also be typed by restriction fragment length polymorphism analysis of amplicons from different regions of the viral genome (21), including the 5'NCR (8, 30). Identification of major HCV types can also be carried out by hybridization of amplicons from the 5'NCR to radiolabelled probes specific for genotypes 1, 2, and 3 (13).

All of these procedures are technically complex, time-consuming, and expensive and therefore not readily applicable to routine use. In addition, PCR-based HCV genotyping cannot be performed in cases with very low levels of viremia or if HCV RNA has been degraded by inadequate handling of samples. To overcome these inconveniences, efforts have been made to identify antibodies against type-specific HCV epitopes by simple serological techniques. Machida et al., by using peptides derived from the core region, were able to distinguish type 1 and 2 HCV infections, but this technique did not provide information in a relatively high proportion of the subjects (20). More recently, Simmonds et al. were able to identify HCV types 1, 2, and 3 in up to 90% of the patients examined with an ELISA using NS4 peptides derived from identified type-specific immunodominant epitopes (31).

Since the laboratory procedures for HCV typing are very heterogeneous, a comparative analysis of the specificity and sensitivity of these methods may provide useful information. To this purpose, the HCV genotype was investigated by three methods in a relatively large series of patients with chronic HCV infection.

In the current study, the type-specific PCR and type-specific hybridization were very sensitive. ELISA, which was able to identify the infecting genotype in 85% of the cases, was somewhat less sensitive. Interestingly, type-specific hybridization achieved high sensitivity despite the use of digoxigenin-labelled probes instead of the radiolabelled probes originally described (13).

Why some sera cannot be genotyped by ELISA is poorly understood. In this study, antibodies against NS4 and NS5 peptides were not detected by the Chiron RIBA HCV 3.0 assay in 5 of the 10 samples that were nonreactive in the ELISA,

suggesting that humoral response against some antigenic regions of HCV may be weak or absent in some patients. In contrast, ELISA-reactive but nontypeable sera showed strong reactivity against the peptides present in Chiron RIBA HCV 3.0 strips. The reasons why HCV cannot be specifically typed by ELISA in reactive sera are unknown.

Another key point is to ascertain whether data from different genotyping methods are comparable or not. The three procedures evaluated in this study showed concordant genotyping results in almost all of the cases in which the type-specific PCR detected a single genotype. In those cases in which the type-specific PCR suggested infection by two or more genotypes, the ELISA and probe-specific hybridization detected the genotype that produced the most intense band in type-specific PCRs. Results concordant among the three methods were observed irrespectively of the genotype involved in each case.

As in this study, concordance between different genotyping methods that focus on different regions of the HCV genome has been reported by others (19). These observations suggest that the sequence heterogeneity defining each genotype is maintained along different regions of the HCV genome (8, 32).

Analysis of epidemiological data showed marked differences between patients with single infections and those with apparently mixed infections suggested by type-specific PCR. A history of IVDU was rare in the former, whereas it was present in one-half of the latter. Repeated exposure to HCV may favor infection by different HCV strains. Furthermore, the marked differences in genotype distribution between patients with single and apparently mixed infections suggested by the type-specific PCR may reasonably be attributed to differences between the patterns of exposure to infection of patients from these two groups.

However, a unique HCV type was detected by ELISA or by type-specific hybridization in the majority of the samples in which mixed genotypes were detected by the type-specific PCR. A possible explanation may be the generation of additional DNA bands by the type-specific PCR. Nonspecific annealing of primers might produce amplicons of different sizes despite the presence of a single HCV cDNA template. In fact, it has been reported that the primer specific for type 1b may react with type 1a, giving rise to false-positive 1a-plus-1b mixed amplifications (1). Such falsely positive reactions have been frequently observed in patients from Canada, where infection with type 1a is highly prevalent (1), and also in European patients (14, 17). Giannini et al. observed that some 1a-plus-1b and 1b-plus-3a apparently mixed infections detected by type-specific PCR in European patients were disclosed as 1a, 1b, or 3a single infections by LiPA typing (14).

On the other hand, several reasons might support the idea that nonspecific annealing of a type 1b primer to type 1a cDNA does not explain why a relatively large proportion of patients in this study appeared to harbor a 1a-plus-1b mixed infection. First, type 1a HCV infection is relatively uncommon in Mediterranean countries (22, 26). Second, a type-specific PCR was carried out with a mixture of the five type-specific primers, which creates competition between PCR reagents and favors the specificity of the reaction (24). Finally, addition of TMAC to PCR mixtures seems to enhance annealing specificity. When cDNAs from three patients infected with type 1a alone were studied by an identical type-specific PCR procedure without addition of TMAC to the amplification mixture, bands of 57 and 144 bp, corresponding to genotypes 1a and 1b, were consistently obtained (data not shown). In oligonucleotide hybridization experiments, it has been shown that addition of TMAC abolishes the preferential melting of AT versus CG base pairs

and makes stringency dependent on the length of the probe (38). In PCRs, addition of TMAC at a low concentration dramatically reduces nonspecific priming events and increases the specificity of the reaction with no inhibitory effect on *Taq* DNA polymerase activity (15).

There are several alternatives to explain the relatively high rate of mixed infection detected by the type-specific PCR. The presence in a given sample of HCV genotypes other than 1a or 1b or those genotypes not detectable by type-specific PCR (such as 1c, 3b, 3c, 4a, 5a, or 6a, occasionally present in Europe), can produce nonspecific priming and weak extra DNA bands. Another explanation may be that sequence heterogeneity of the core region present in European type 2 isolates can lead to mistyping by type-specific PCR (14, 17). This possibility is supported by the current study, in which type 2 was always detected as part of apparently mixed infections despite the use of a modified primer to detect Mediterranean type 2a and 2c isolates. Finally, the observation that type 3a was detected as part of apparently mixed infections in 8 of 18 cases suggests that the variability of the core region might also produce nonspecific bands when samples containing type 3a are analyzed by type-specific PCR.

The possible lack of specificity of the type-specific PCR in HCV genotyping studies can only be ruled out with certainty by sequence analysis of an appropriate number of clones derived from the amplified PCR product. In four cases with apparently mixed infections, as suggested by type-specific PCRs, a unique HCV type was identified by 5'NCR sequence analysis of at least 10 clones from each patient. These observations suggest that nonspecific priming is not completely prevented by addition of TMAC to the type-specific PCR mixture. Patients with past IVDU are more likely to be infected with genotypes other than 1 (22), and this may favor overestimation of mixed infection by a type-specific PCR. Therefore, the epidemiological association of mixed infection with a history of IVDU observed in this study might be an artifact.

Theoretically, in true mixed infections, one of the genotypes might be represented in an extremely large proportion with respect to the others. Therefore, nonpredominant types present in a mixed viral population could be underestimated by analysis of only 10 clones. However, nonpredominant types might be detected by the type-specific PCR, which is extremely sensitive. Investigation of a very large number of clones may be required to confirm whether a mixed infection detected by the type-specific PCR is true or not.

In summary, the highly concordant genotyping results of this study suggest that the three laboratory techniques evaluated may reliably be used in genotyping studies of HCV. Each procedure has advantages and drawbacks. ELISA is rapid and simple and, in contrast with other genotyping methods, does not require the strict conditions necessary for HCV RNA preservation in serum samples. However, ELISA is somewhat less sensitive and does not allow identification of HCV subtypes. Probe-specific hybridization, which is more complex and time-consuming, offers greater sensitivity for identification of major genotypes. The type-specific PCR, as used in this study, is also complex and demands great care for specificity but provides virus subtype information, in contrast to the other procedures. Detection of a mixed infection by a type-specific PCR is relatively frequent and should be interpreted with caution.

ACKNOWLEDGMENTS

This work was supported in part by grant 94/848 from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad. X. Fornés is the recipient of an Emilio Letang grant from the Hospital Clínic i Provincial, Barcelona, Spain. F. X. López-Labrador is supported by the Spanish

Ministerio de Educación y Ciencia (grant FPI-AP91/43508114). S. Ampurdanés and E. Olmedo are supported by the Fundació Catalana per l'Estudi de les Malalties del Fetge.

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